Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 147-152

INDUCTION OF F9 CELL DIFFERENTIATION BY TRANSIENT EXPOSURE TO RETINOIC ACID

Jing-Ming Dong, Feng Li and Jen-Fu Chiu

Department of Biochemistry, University of Vermont
College of Medicine, Burlington, Vermont 05405

Received May 17, 1990

The F9 cell is a mouse embryonal teratocarcinoma which can be induced to differentiate into visceral endoderm by treatment with retinoic acid (RA). Treatment with RA in conventional studies was carried out in the constant presence of RA. Here we demonstrate that treatment with RA can be as short as 3 hrs to induce differentiation of F9 cells. Morphology, alpha-fetoprotein gene activity, and temporal patterns of F9 cell differentiation are the same with both short- and long-term treatment with RA.

Embryonal carcinoma cells (EC cell) are multipotential stem cells of a teratocarcinoma. Upon several types of physical and chemical stimuli, EC cells can differentiate into somatic tissue corresponding to derivatives of three germinal layers, namely endoderm, mesoderm, and ectoderm (1,2). One of the most extensively studied EC cell lines is the F9 cell. This cell line was originally derived from a testicular tumor that arose spontaneously in the gonads of 129/SJ mouse (3). F9 cells do not undergo significant spontaneous differentiation and are highly malignant (4). It has been shown that F9 cells treated with retinoic acid (RA) give rise to primitive endoderm (5) and when these cells are treated with dibutyl cyclic AMP, they differentiate further into parietal endoderm(6). When RA treated F9 cells are allowed to grow in suspension to form aggregates, most of the cells on the outer surface will differentiate into visceral endoderm which possess an ability to producing α-fetoprotein(AFP) (5). The capacity of F9 cells to differentiate in a controlled manner in culture provides a useful model system for studying early events of mammalian development and differentiation.

The conventional approach to induction of F9 cells differentiation is treatment of cell cultures with RA for at least 6 to 8 days (7,8). Here we demonstrate that RA treatment can be as short as 3 hours. Based on morphological examination and temporal patterns of AFP gene activity, the differentiation pattern of F9 cells are the same with both short- and long-term treatment of RA. These data suggest that RA acts as an initiator to trigger the intracellular cascade events of F9 cell differentiation.

0006-291X/90 $1.50

MATERIALS AND METHODS

Cell culture and differentiation. Undifferentiated F9 cells, obtained from Dr. Eileen Adamson, were maintained as stem cells by culturing them on gelatin-coated tissure culture flasks in a minimal essential medium with nucleotides (Irvine Scientific), supplemented with 10 % fetal bovine serum, and 1 mM glutamine. For the experiments on cell differentiation, F9 cells were seeded at 5 x 10^5 cells per 100-mm bacterological petri dishes in a 50 : 50 mixture of Dulbecco Modified Eagle Medium with a high concentration of glucose and Ham F-12 Medium (GIBCO) supplemented with 10 % fetal bovine serum, and 1 mM glutamine. On the day after seeding, the cell aggregates were treated with 5 X 10^{-8} M all trans-RA (Sigma) for designated periods of time as stated. The RA was then removed by collecting cell aggregates in a 15 ml conical tube and washing the cell pellet 2 times with 5 ml fresh medium. The cell aggregates were then refed with fresh medium without RA for 9 days. The medium was changed every day. Undifferentiated F9 cell were also grown as aggregates in petri dishes without RA.

Total RNA preparation and Northern analysis. cells were lysed by the guanidinium thiocyanate, and total RNA were prepared by the procedure of Maniatis et al (9). RNA samples were electrophoretically separated on formaldehyde-agarose gel as described (10). The gels were transferred to nitrocellulose in 20 X SSC (1X SSC = 0.15 M NaCl, 0.0015 M sodium citrate). The prehybridization, hybridization, and washing procedures were conducted according to Thomas (11). The DNA probes used are pRAF-87 (AFP cDNA) or pA1 (B-Actin cDNA) labeled with ^{32}P-dCTP by the random priming procedure (12).

RESULTS

Figure 1A shows a monolayer culture of undifferentiated F9 cells. F9 cells formed cell aggregates with a smooth ring surface. They did not show any cell differentiation or morphological changes when cultured in suspension for 9 days in the absence of RA (Fig. 1B). Following RA stimulation, F9 cells commit to differentiation. The cell aggregates start to form embryoid bodies with a ring of outer cells which resemble visceral endoderm cells at day 3-5 (the day RA was added is designed as day 0) (13). These outerlayers of the embryoid bodies have a rough surface while F9 cell aggregates grown in suspension under the same conditions without RA treatment have a smooth surface (Fig. 1B). No distinquishable difference in morphology can be detected in the cells treated with RA for either a long term (constantly present in media) or a short term (3 hours) (Fig. 1C).

Biochemically, visceral endoderm is characterized by its ability to produce AFP (6,14). As shown in Fig. 2, after transient treatment with RA for as short as 3 hours, a low level of AFP gene expression can be detected in the cell culture at day 9. The amount of AFP mRNA in the cells treated with RA for 24 hours increased to the same level as in the cells with constant treatment of RA. No significant change in β-actin gene expression was observed. The data suggest that the time required for RA to initiate F9 cell differentiation is quite short. RA does not have to be present in the medium after it triggers the first step of the differentiation cascade.

To further study if RA can activate genes whose protein products are involved in the process of F9 cell differentiation, the cells were pre-treated

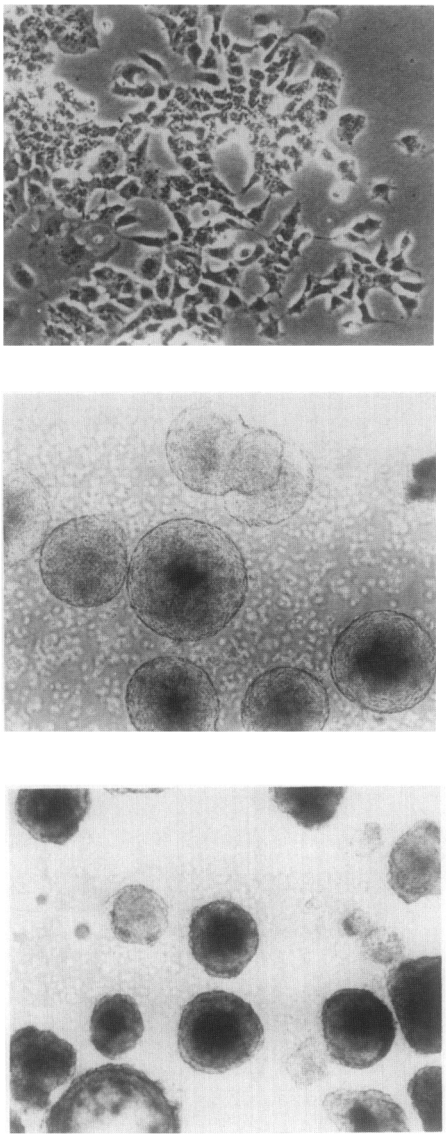

Figure 1. The morphorlogy of differentiated F9 cell aggregates after transient treatment with RA. A. Undifferentiated F9 stem cells grown as a monolayer of cells. B. Undifferentiated F9 cell aggregates in suspension cultured for 9 days without RA. C. Differentiated F9 cell aggregates treated with RA for 3 hrs, and after washing out RA, cells were continuely cultured up to 9 days. Magnification X 100.

with the protein synthesis inhibitor cycloheximide for 2 hours before treatment with RA. F9 cells were then treated with RA plus cycloheximide (15) for 6 hrs. At the end of treatment, cells were washed and continuously cultured in fresh media for up to 9 days. The cells appear as cell aggregates with smooth surface similar to the morphology as shown in Fig. 1B. No cell differentiation was observed in the cells at day 9 and no AFP gene expression can be detected (Fig.3

lane 1). Similar results were obtained when cells were treated with RA for 6 hrs followed by treatment with cycloheximide for 8 hrs (Fig.3 lane 2). Cycloheximide treatment alone does not induce cell differentiation and AFP gene expression (Fig.3 lane 4). The data indicate that the protein product(s) of gene(s) which respond to the treatment of RA play a crucial role in the process of F9 cell differentiation.

The induction of AFP gene expression is a late event in F9 cell differentiation. Some stage-specific factor(s) needed for activation of AFP gene expression were synthesized during the cascade of events in differentiation. In order to study when these factor(s) were synthesized, the time course of the cycloheximide effect was examined. One day after treatment with RA, treatment

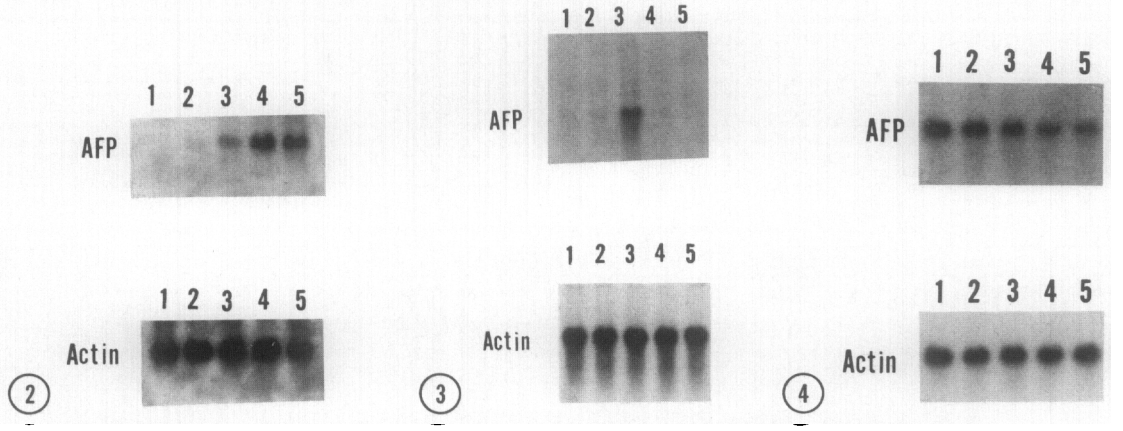

Figure 2. Expression of the AFP gene in F9 cells transiently treated with RA. F9 cell aggregates were cultured in the presence of 5 X 10^{-8} M RA for 0 hr (lane 1), 3 hrs (lane 2), 6 hrs (lane 3), 24 hrs (lane 4) and 9 days (lane 5). After washing out of RA, cells were continuously cultured up to 9 days. Total RNA was isolated and 10 μg of total RNA from each sample was used for Northern blot analysis.

Figure 3. The effect of cycloheximide on AFP gene expression in differentiated F9 cells. Lane 1: Cells were first cultured in media with 2 ug/ml cycloheximide for 2 hrs, RA was then added to media at a final concentration of 5 X 10^{-8} M and incubation continued for 6 hrs; Lane 2: Cells were cultured in the presence of 5 X 10^{-8} M RA for 6 hrs. Cells were then washed to remove RA, and further incubated in fresh media containing 2 ug/ml of cycloheximide for 8 hrs; Lane 3: Cells were cultured in 5 X 10^{-8} M RA for 6 hrs; Lane 4: Cells were cultured in media containing 2ug/ml of cycloheximide for 8 hrs; Lane 5: F9 cell aggregates were cultured without RA for 9 days. At the end of treatment as described above, Cells were washed and continuously cultured in fresh media for up to 9 days. 10 μg of total RNA from cells in each experiment were analyzed by Northern blot.

Figure 4. Time course of the cycloheximide effect on AFP gene expression in F9 cells after 1 day treatment with RA. F9 cell aggregates were cultured in 5 X 10^{-8} M RA for 24 hrs. Cells were washed to remove RA and then cultured in fresh media with or without cycloheximide for 24 hrs at various time points. Cells were washed again to remove cycloheximide and continuously cultured in fresh media for up to 9 days. Lane 1: no cycloheximide treatment; Lane 2: cycloheximide treatment for 24 hrs at day 2; Lane 3: cycloheximide treatment for 24 hrs at day 3; Lane 4: cycloheximide treatment for 24 hrs at day 4; Lane 5: cycloheximide treatment for 24 hrs at day 5. 10 μg of total RNA from cells of each experiment were analyzed by Northern blot.

with cycloheximide for 24 hrs has no effect on AFP gene expression at day 9 (Fig. 4). Similarly, at any period of time thereafter during cell differentiation, cycloheximide has no significant effect on AFP gene expression (Fig. 4). These data suggest that the RA responsive protein factor(s) involved in the process of F9 cell differentiation were synthesized within 24 hrs after treatment with RA, and that the production of trans-acting factor(s) responsible for induction of AFP gene expression are not affected by cycloheximide after RA responsive gene(s) were turned on.

DISCUSSION

RA, which belongs to a class of polyisoprenoid lipid molecules, is structurally and functionally related to vitamin A. RA can affect the proliferation and differentiation of a variety of cell types (16,17), suppress carcinogenesis in vivo (16,17), and affect pattern formation during development (18,19). The molecular mechanism by which RA exerts these profound effects on differentiation, growth, and development has not been fully elucidated. Presumbly the mechanism should involve the regulation of expression of specific genes.

There are two pieces of evidence in this paper to suggest that RA acts as an inducer to trigger the differentiation cascade of F9 cells. First, the transient treatment of RA can induce F9 cells to aggregate and differentiate into visceral endoderm which exhibits characteristic AFP gene expression. This indicates that RA only acts as an initial signal. Secondly, the differentiation of F9 cells can be blocked by a protein synthesis inhibitor during the first 6 hrs of RA induction. This suggests that the differentiation cascade is turned on by the activation of RA responsive gene(s). The products of these genes are most likely trans-acting factors which play an important role in modulating expression of other genes involved in the differentiation process of F9 cells. Recently, Gudas's group isolated a cDNA clone named ERA-1 from F9 cells committed to differentiation into parietal endoderm in response to RA treatment (20). The expression of the ERA-1 gene is promptly induced upon addition of RA to cell culture. The sequencing data shows that the product of this gene contains a homeobox region (21) and is likely to encode a DNA-binding protein which can further regulate the secondary response genes. It is unclear whether this gene is also involved in the visceral endoderm differentiation pathway. Following the addition of RA to mouse macrophage cell culture, Chiocca et al (22) found a rapid and transient induction of the transglutaminase gene. The level of transgluminase mRNA increased ten fold 3 min after treatment with RA and reached the highest level by 10 hrs. The role of transgluminase in cell differentiation is not known.

Our observations indicate that the presence of cycloheximide at day 2 for 24 hrs has no effect on the level of AFP gene expression at day 9 and that RA

could be washed out after 3 hrs with little effect on AFP mRNA accumulation at day 9. These results suggest that the synthesis of protein factors controlling the differentiation process should be completed by day 2 although the induction of AFP gene expression occurs much later. Our data also suggest that the trans-acting factors needed for the activation of the AFP gene have been produced by day 2 following initiation of F9 cell differentiation with RA, and that these trans-acting factors are constantly present in the cells for the next few days, because after day 2 the inhibition of protein synthesis at any time point has little effect on AFP mRNA level at day 9 (Fig.4). However we can not rule out the possibility that the AFP gene suppressor disappeared upon the induction of F9 cell differentiation by RA.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Eileen Adamson for kindly providing F9 cell lines, Dr. Thomas Sargent for cloned AFP gene pRAF87, Dr. Don Cleveland for cloned β-actin gene pA1. We also wish to thank Jack Egan for critical reading of this manuscript.

REFERENCES

1. Dixon,F.J.,and Moore,R.A. (1953) Testicular tumors clinicopathologic study Cancer 6, 451-454.
2. Jetten,A.M. (1985) In Retinoids and cell differentiation (M.I. Sherman,Ed.). CRC Press, Boca Raton, FL.
3. Artzt,K., Pubois,P., Bennett,D., Condamine,H., Babinet,C., and Jacob,F. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2988-2992.
4. strickland,S. (1981) Cell 24, 277-278.
5. Strickland,S., and Mahadavi,V. (1978) Cell 15, 494-403.
6. Sherman,M.I., and Miller,R.A. (1978) Dev. Biol. 63, 27-34.
7. Hogan,B.L.M., Taylor,A.,and Adamson,E.D. (1981) Nature 291, 235-237.
8. Grover,A., and Adamson,E.D. (1986) Dev.Biol. 114, 492-503.
9. Maniatis,T.,Fritsch,E.F., and Sambrook,J. (1982) Molecular cloning: a laboratory manual. Cold spring Harbor laboratory, Cold spring harbor, N.Y.
10. Zhang, X.K., Wang, W., Lee, A., Huang, D.P., and Chiu, J.F. (1988) Cancer Lett. 41, 147-155.
11. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5201-5205.
12. Feinberg,A.P., and Vogelstein,B. (1983) Anal. Biochem. 132, 6-13.
13. Adamson,E.D., and Grover,A. (1983) In: Teratocarcinoma stem cells (L.M. Silver, G.R. Martin, S. Strickland, Eds), Vol. 10, pp.69-81 Cold spring harbor Laboratory, N.Y.
14. Dziadek,M., and Adamson,E. (1978) J.Embryol.Exp. Morphol. 4, 289-15.
15. Grollman,A.P. (1966) Proc. Natl. Acad. Sci. U.S.A. 80, 5525-5529.
16. Roberts,A.B., and Sporn,M.B. (1984) pp.210-286. In The retinoids (M.B. Sporn, A.B. Robert, and D.S. Goodman, eds.) Vol.2, pp. 210-286. Academic Press, Inc. N.Y.
17. Sporn,M.B., and Robert,A.B. (1983) Cancer Res. 43, 3034-3040.
18. Tickle,C., Lee,J., and Eichele,G. (1985) Dev. Biol. 109, 82-85.
19. Thaller,C., and Eichele,G. (1987) Nature 327, 625-628.
20. Larosa,G.J., and Gudas,L.J. (1988) Proc.Natl.Acad.Sci.U.S.A. 85, 329-333
21. Larosa,R.J., and Gudas,L.J. (1988) Mol. Cell. Biol. 8, 3906-3817.
22. Chiocea,E.A., Davies,P.J.A., and Stein,J.P. (1988) J.Biol.Chem. 263, 11584-11589.